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Generating improved single-chain Fv molecules for tumor targeting

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Abstract

Due to their ease of isolation from phage display libraries and their ability to recognize conserved antigens, single-chain Fv (scFv) molecules are rapidly becoming commonplace. However, the monovalent nature of the scFv molecule often dictates, at best, transient interactions with target antigens when molecules with moderate to low affinity are employed. This, along with their rapid elimination from circulation, has limited the utility of scFv molecules for applications in the fields of cancer imaging and therapy. Recently, a number of strategies, including affinity maturation and modification of size and valence, have been evaluated for improving the in vivo efficacy of scFv molecules. In this review, we describe a number of these methods and discuss some of the characteristics that may belong to an optimal antibody-based targeting vehicle.

Keywords: Single-chain Fv (scFv); Affinity; Phage display; Mutagenesis; Valence

1. Introduction

In recent years, monoclonal antibodies (MAbs) have become accepted tools for the detection and treatment of cancer. In the United States, the Food and Drug Administration (FDA) has approved the commercial sale of MAb-based radioimmunodetection (RAID) systems for colon cancer (Oncoscint®) and prostate cancer (Prostascint®). Unmodified antibodies have also been approved for the treatment of lymphoma (Rituxan®) and breast cancer (Herceptin®), and the first commercial radioimmunotherapy (RAIT) drug (Bexxar®) is expected to be licensed for the treatment of lymphoma. With the expectation of similar approvals worldwide and a long pipeline of antibody-based drugs in clinical trials, MAbs are finally living up to their proposed potential. However, the current generation of MAb-based drugs that is entering the clinic is limited by the physical characteristics of the MAb. Immunoglobulin molecules have evolved to recognize and clear foreign pathogens. In humans, multiple exposures to a specific antigen drives the affinity maturation of the humoral immune response, generating variants of the initial immunoglobulin molecules with improved capabilities to bind and clear the target. Antigen binding activity is determined by the sequences and

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conformation of the amino acids of the six complementarity determining regions (CDRs) that are located (three each) on the light and heavy chains of the variable portion (Fv) of the antibody. Sequences in the constant region (Fc) interact with a variety of proteins and cells that perform numerous immune functions (e.g., elimination of circulating pathogens, fixation of complement, targeting of the innate immune response) (Deo et al., 1997) and regulate the turnover of Ig molecules in circulation (reviewed in Ghetie and Ward, 1997). With a molecular weight of approximately 155 kDa, antibodies exhibit a prolonged availability in circulation that can lead to bone marrow exposures that are associated with unacceptable myelotoxicities when RAIT strategies are employed. The large size of the intact MAb also limits the ability to diffuse from vasculature into a tumor due to the high hydrostatic pressure resulting from the disordered blood vessels and the lack of draining lymphatics (Jain and Baxter, 1988). Furthermore, interactions with the Fc receptors (FcR) located on normal tissue can alter the distribution of the MAb, potentially endangering the patient when toxic payloads are attached (e.g., radioisotopes or catalytic toxins). Clearly, these characteristics limit their utility as vehicles for the treatment of solid tumors.

In order to address the limitations of large IgG molecules, smaller engineered MAb-based molecules were developed. The 25-kDa single-chain Fv (scFv) molecule, composed of a variable region (V_L) of the light chain and a variable region (V_H) of the heavy chain joined via a short peptide spacer sequence, is the smallest antibody fragment developed to date with potential clinical applications. While smaller species have been generated (e.g., V_H domains and peptides containing a complementarity determining region [CDR]), they suffer from both a low affinity for their target antigen and a tendency to cross-react with unrelated antigens (reviewed in Adams, 1998). The first scFv molecules were developed independently by Huston et al. (1988) and Bird et al. (1988). These molecules, derived from genes isolated from murine hybridoma cell lines, were capable of specifically binding to their target antigens with affinities ranging up to those of their parent MAbs. More recently, techniques have been developed to display scFv molecules on the surface of filamentous phage that contain the gene for the scFv. scFv molecules with a broad range of antigenic-specificities can be present in a single large pool of scFv-phage complexes (phage library). The genes for antigen-specific scFv can be readily isolated (by panning against the antigen) and used to express the desired scFv in *Escherichia coli* (Schier et al., 1995).

In vivo, anti-tumor scFv molecules display rapid, biphasic pharmacokinetics. Their $T_{1/2}\alpha$ (equilibration phase) is between 2 and 12 min, while their $T_{1/2}\beta$ (elimination phase) is typically about 1.5 to 4 h (reviewed in Adams, 1998). As they quickly equilibrate, the scFv readily penetrate into solid tumors (Yokota et al., 1992). However, their rapid renal elimination limits the quantitative localization of scFv in the tumor (Colcher et al., 1990; Milenic et al., 1991; Adams et al., 1993). A number of modification strategies has been successfully employed to improve the localization of scFv-based molecules in tumors. Raising the intrinsic affinity of an scFv for its target by modifying the CDRs (antigen-contact regions), increasing the functional affinity (avidity) of the scFv through the creation of multivalent species and/or increasing the size of the scFv such that it falls near or above the threshold for renal uptake (approximately 60–70 kDa) (Chang et al., 1975; Maack et al., 1979) can all lead to increased localization in tumors. Here, we will discuss strategies for increasing the affinity and improving the tumor targeting of scFv-based molecules.

2. Affinity of scFv generated from hybridoma cell lines

When generating a scFv from the immunoglobulin variable region genes isolated from a hybridoma cell line, a decrease in the apparent affinity of the resulting scFv as compared to the parental MAb has often been observed (Huston et al., 1988). With many scFv molecules, the lower affinity results from the decrease in valence (number of binding sites) that occurs when the format is switched from the larger bivalent MAb to the smaller monovalent scFv. This is particularly true when multiple copies of the target epitope are present on a single antigen molecule as was the case with the CC49 MAb

(Milenic et al., 1991). CC49 is specific for a repeated clustered carbohydrate epitope on the TAG-72 pancarcinoma antigen. CC49 MAb binds to TAG-72 with an apparent affinity of 2×10^{-9} M, 8-fold greater than that observed with the engineered scFv and enzymatically prepared Fab′ molecules. The similarity between the affinities of the CC49 scFv and Fab′ indicates that the change from a dimeric to a monomeric format is the most important factor in the decrease in affinity. However, this phenomenon is dependent upon the expression and availability of the epitope on the target antigen. For example, the affinities of the IgG, Fab′ and scFv forms of B6.2, specific for a 90,000 kDa protein related to the carcinoembryonic antigen (CEA) gene family, are very similar (2.3×10^{-9}, 3.8×10^{-9} and 3.1×10^{-9} M, respectively) (Colcher et al., 1990). Decreases in affinity can also result from structural alterations between the IgG and scFv formats. In particular, the peptide spacer that joins the V_H and V_L chains can potentially interfere with the normal alignment of the two chains. In general, since the production of scFv from hybridoma-derived genes can be associated with a loss of affinity, it is best to start with the highest affinity parental MAb available.

3. scFv from phage display libraries

A number of factors can influence the quality and quantity of scFv isolated from phage display libraries. The characteristics of the scFv repertoire library (size, diversity and functionality), the origin of the library (naive, immune or synthetic) and the selection strategy employed are the most critical parameters. Large antibody repertoire libraries ($> 10^9$ transformants) of a naive origin (i.e., genes from non-immunized donors) usually provide a wider variety of scFv specificities and higher affinities than do smaller antibody repertoire libraries (e.g., 10^7 transformants) of the same origin. This was demonstrated in experiments in which we employed HER2/*neu* extracellular domain (ECD) as a target for selections in small (Marks et al., 1991; Schier et al., 1995) and large naive antibody libraries (Sheets et al., 1998). Selections performed in the large library yielded 14 different scFv with K_D as low as 2.2×10^{-10} M while selections performed in the small library resulted in the isolation of only two distinct scFv clones with two log poorer affinity (K_D of 1.6×10^{-8} M). In general, affinities of scFv isolated from small naive libraries are comparable to the affinities of antibodies generated in primary in vivo immune responses (e.g., 10^{-6} to 10^{-8} M) (reviewed in Winter et al., 1994). In contrast, scFv isolated from larger libraries (e.g., 10^9 to 10^{11} specificities) often have affinities in the range of antibodies produced in a secondary or tertiary immune response (e.g., 10^{-8} to 10^{-10} M) (Griffiths et al., 1994; Winter et al., 1994; Vaughan et al., 1996; Sheets et al., 1998). However, it is important to recognize that the functional size of a library can be smaller than the calculated size. The percentage of the scFv genes that are expressed and result in the display of functional protein on the surface of the phage can be lower than expected due to misfolding resulting from promiscuous V_L–V_H chain pairing.

In regard to the origin of the library, the diversity of the repertoire is limited by natural boundaries of the gene repertoire of the donors. Immune antibody libraries are typically derived from splenocytes of mice that have been previously immunized with the intended target antigen (Clackson et al., 1991). As a result, these libraries are enriched for genes coding for the variable domains of antibodies specific for the desired antigen target (Winter et al., 1994). In contrast, naive antibody libraries typically contain both smaller quantities of specific scFv and a narrower range of affinities since the genes used for generating these libraries have not already undergone cycles of somatic hypermutation. Semi-synthetic scFv libraries are composed of V_L and V_H genes with randomized CDRs (Hoogenboom and Winter, 1992; Nissim et al., 1994; de Kruif et al., 1995a). These can surpass the diversity of naive repertoires by adding additional sequences that are not naturally available. However, randomizing the stretches of CDR sequence can lead to a reduction of overall functionality of expressed and displayed scFv pools, resulting in a negative influence on the quality and quantity of the selection process.

The choice of selection strategy has a major impact on the outcome of the isolation process which

is independent of the nature of the repertoire. Antigen presentation, washing conditions, incubation periods and elution conditions employed during the panning procedure are all critical parameters that determine the success of scFv selection. Employing solid supports (e.g., Immunotubes) for an antigen target can alter the conformation of the antigen and lead to the isolation of scFv that recognize only unnatural or denatured forms of the antigen (Schier et al., 1995). This concern can be addressed by selecting in solution using biotinylated antigen and recovering specific binders with streptavidin-conjugated magnetic beads (Hawkins et al., 1992). This technique is highly recommended when the isolated scFv will be used for in vivo targeting of cell-surface antigens (e.g., tumor-associated antigens). Furthermore, the isolation of higher affinity scFv can be promoted by using this approach while limiting antigen concentrations during the course of panning (Schier et al., 1996a,b; Sheets et al., 1998). Here, the concentration of antigen employed is particularly important. In the first round of selection, a higher concentration should be used in order to capture rare or poorly expressed phage antibodies. However, in subsequent rounds of an affinity selection the concentration of antigen should be decreased so that it is significantly lower than the desired K_D and less than the concentration of phage present. Alternatively, when a biotinylated antigen system is employed, lower affinity clones (with faster off rates) can be eliminated by adding excess non-biotinylated antigen after an initial incubation with the biotinylated antigen. The use of the biotinylated antigen/streptavidin magnetic-bead technique also reduces the avidity advantage that spontaneously dimerizing scFv have over monomeric scFv, again favoring the isolation of high affinity monomeric scFv (Schier et al., 1996a). Cell lines or tissues expressing cancer-associated antigen on their surface can also be employed for phage selection (de Kruif et al., 1995b; Pereira et al., 1997; Tordsson et al., 1997; Figini et al., 1998). This approach may increase the likelihood of identifying scFv that bind a cell receptor in the context of the cell membrane. Finally, washing conditions, incubation times and elution conditions drive the panning experiment (unpublished data). Low stringency conditions favor the enrichment of scFv that are extremely well expressed whereas high stringency shifts the selected scFv pool towards higher affinity.

4. Valence of engineered antibodies

As noted above, a major limitation of scFv molecules is their monovalent interaction with target antigen. Logically, one of the easiest methods of improving the binding of a scFv to its target antigen is to increase its functional affinity through the creation of a multimer. A number of multivalent scFv-based structures has been engineered, including miniantibodies (Pack and Plückthun, 1992), dimeric miniantibodies (Müller et al., 1998), minibodies (Hu Shi-zhen et al., 1996), $(scFv)_2$ (Adams et al., 1993), diabodies (Holliger et al., 1993) and triabodies (Iliades et al., 1998; Lawrence et al., 1998; Hudson and Kortt, 1999) (Fig. 1). These molecules span a

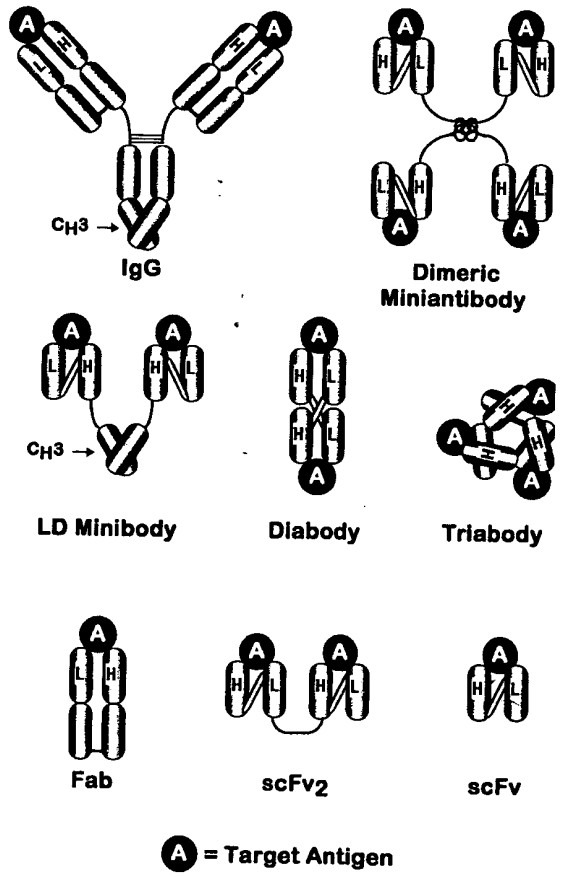

Fig. 1. Antibody structures. Schematic structures of a variety of antibody-based molecules ranging in size from ~25 to ~155 kDa and valence from one to four binding sites.

range of valence (two to four binding sites), size (50 to 120 kDa), flexibility and ease of production.

Among the easiest constructs to engineer are the noncovalent diabody and triabody molecules. These are produced by shortening the peptide linker that connects the variable heavy and variable light chains of a single scFv molecule from 15 amino acids to five amino acids (diabody) or 0–3 amino acids (triabody) (discussed in greater detail in Peter Hudson's chapter located elsewhere in this volume). A variety of miniantibodies have been produced by Pack and Plückthun. These scFv dimers are joined by amphipathic helices that offer a high degree of flexibility. Müller et al. (1998) recently modified the miniantibody structure to create a dimeric bispecific (DiBi) miniantibody that contains two miniantibodies (four scFv molecules) connected via a double helix. Gene-fused or disulfide-bonded scFv dimers provide an intermediate degree of flexibility and their production depends upon relatively straightforward cloning techniques (e.g., the addition of a C-terminal Gly_4Cys sequence). Hu Shi-zhen et al. (1996) have created scFv-C_H3 minibodies that are comprised of two scFv molecules joined to an IgG C_H3 domain either directly (LD minibody) or via a very flexible hinge region (Flex minibody). With a molecular weight of approximately 80 kDa, these divalent constructs are only half the size of their parental IgG molecule yet still are capable of significant binding to cell surface antigens. The Flex minibody, in particular, exhibits impressive tumor localization in immunodeficient mice. However, the use of glycosylated IgG C_H3 domains in the minibodies necessitates their production in mammalian cell lines (e.g., Sp2/0 murine myeloma cells). This process is significantly more difficult than the *E. coli* expression methodology for the other multimeric species described above. The appropriate structure for a given application is dependent upon both target antigen orientation and the requirements for effective targeting. For example, the optimal compound for use in an in vitro ELISA assay for the detection of serum proteins could be large and multivalent with limited flexibility (e.g., a triabody). However, the requirements for effective in vivo targeting of a cell surface antigen in a solid tumor may favor smaller divalent constructs with a greater flexibility (e.g., a minibody or a $scFv_2$).

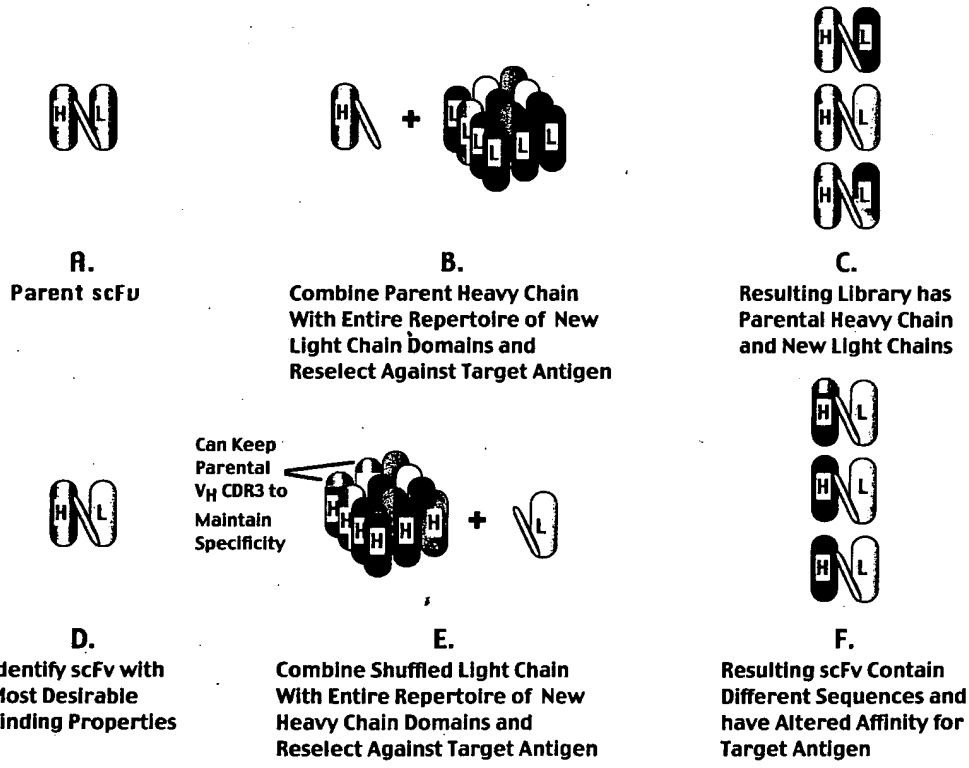

Fig. 2. Chain-shuffling schema. The procedure for enhancing scFv affinity via chain shuffling is illustrated in this diagram.

5. Affinity maturation by chain shuffling

Unlike the dimerization strategies, chain shuffling and site-directed mutagenesis (presented below) involve alterations to the intrinsic affinity of the monovalent antibody fragment. In chain shuffling, the scFv molecule is subjected to cycles of manipulations in which the gene for one chain (e.g., V_H) is cloned into a repertoire for the second chain (V_L) (Fig. 2) (Marks et al., 1992). The resulting library, that contains scFv-phage with V_H chains specific for the antigen and random V_L chains, is panned against the target antigen to identify clones with improved binding characteristics. The cycle is then repeated, this time shuffling the new V_L gene into a V_H repertoire. Since the V_H CDR3 contains most of the contact residues to the antigen, it is advisable to retain this region from the parent scFv to maintain specificity. In this case, only the V_H segment from frameworks 1 to 3, including the CDRs 1 and 2, are replaced. Using this methodology, we increased the affinity of the anti-HER2/*neu* C6.5 scFv 6-fold from 1.6×10^{-8} to 2.6×10^{-9} M (Schier et al., 1996a). Interestingly, most of the relevant alterations that occurred during the shuffling process were found to be in the framework regions of the V_L and V_H chains far away from the actual binding sites. Clearly, these changes could not have been readily predicted or designed. Furthermore, this supports the need for a sequential approach to affinity maturation. Particularly since the enrichment of mutations in the frameworks in a parallel panning process with subsequent combining of optimal V_H and V_L chains may be counterproductive and lead to low affinity scFv (Schier et al., 1996a).

Chain shuffling employs the repertoire of naturally-occurring antibody variable genes displayed on filamentous phage as prospective partners for the sequential ''ratcheting up'' of the antibody's affinity. This method provides additional diversity, particularly in the framework regions of the antibody fragments and hence, the potential for significant increases in affinity. Furthermore, the sequential manipulations that are required to maintain antigen specificity throughout this process make chain shuffling a viable technique for the humanization of murine MAbs (Rader et al., 1998). For this application, the mouse V_L and V_H chains are in turn shuffled into human repertoire libraries. However, a potential drawback associated with chain shuffling is that it can result in changes to the antigenic specificity of the scFv (Ward, 1995).

6. Affinity maturation by site-directed mutagenesis

This method entails substitutions of the amino acids of one or more of the CDRs and the subsequent selection of clones with higher affinity for the antigen. We have employed this approach to modify the affinity of C6.5, a scFv that binds to HER2/*neu* with a moderate affinity (K_D 1.6×10^{-8} M) (Schier et al., 1996b). In this case, both V_H and V_L CDR3s were sequentially randomized. The V_L CDR3 repertoire we employed was designed to conserve the wild type amino acid in each position at a frequency of 49%. A 16-fold increase in affinity was achieved (C6ML3–9; K_D 1.0×10^{-9} M) via randomization of the light chain CDR3. The resulting affinity mutant with the greatest affinity, C6ML3–9, was used as the starting molecule for the manipulation of the heavy chain. Four V_H CDR3 libraries were generated, each containing groups of four randomized amino acids. Selections performed in the presence of limiting amounts of biotinylated antigen yielded affinity mutants with K_D of up to 1×10^{-10} M. By combining the mutations isolated from two of the four V_H CDR3 libraries, the resulting affinity was increased to 1.3×10^{-11} M (approximately 1200-fold improvement over the parental scFv). Site-directed mutagenesis can also be effectively used to decrease the affinity of a scFv. Sequential alanine scanning of the V_H CDR3 was used to identify potentially important contact residues. This yielded scFv with measurable affinities of as low as 10^{-6} M for HER2/*neu* and some scFv affinity mutants that were incapable of binding to HER2/*neu* (Schier et al., 1996b). Therefore, using this robust methodology we generated a series of C6.5 scFv-based molecules that ranged in affinity for HER2/*neu* from 10^{-6} to 10^{-11} M (100,000-fold). In recent years, affinity maturation by randomization of V_H and V_L CDR3s in combination with phage display has become a relatively straightforward procedure that has been

employed by many groups (Yang et al., 1995; Thompson et al., 1996; Pini et al., 1997).

A refinement of the above strategy, termed "parsimonious mutagenesis", was originally described by Balint and Larrick (1993). In this method, the entire CDR sequence of an antibody fragment is screened to identify amino acids that are actively involved in the binding to antigen. Two methods are used to improve the efficiency of the selection process. First, the number of codons introduced is limited such that each amino acid is only coded for by a single codon. Second, the amino acids at each position are manipulated to favor parental sequences, conservative changes and those that more commonly appear in antibody CDR regions. Using this system, Schier et al. (1996c) modified the affinity of the anti-HER2/*neu* scFv C6.5 from 1.6×10^{-8} to 2.4×10^{-9} M. As parsimonious mutagenesis provides information about the contribution of distinct amino acids to binding and affinity, the procedure can be useful as an initial step in the affinity maturation process.

Each of the methods described above is likely to lead to the generation of new and distinct affinity mutants. Accordingly, combining strategies may further increase the affinity of a scFv. For example, Osbourn et al. (1996) employed both parsimonious mutagenesis and chain shuffling to increase the affinity of an anti-CEA scFv from 8×10^{-9} to 6×10^{-10} M. In this case, chain shuffling and parsimonious mutagenesis alone resulted in 2- to 3-fold and 4-fold respective improvements in affinity, while combining the alterations from both strategies resulted in a 12-fold improvement in affinity.

What is the theoretical upper limit of affinity? While antibodies in a tertiary immune response typically range in affinity for their target antigens from 10^{-9} to 10^{-10} M, the majority of the binding energy is contributed by about 30% of the 15–22 contact residues (reviewed in Balint and Larrick, 1993). The other residues most likely contribute little binding energy or are "repulsive". Balint and Larrick state that the potential cost of these "repulsive" contacts can equal up to three logs of affinity. Therefore, dependent upon the nature of the target epitope, 10^{-12} to 10^{-13} M affinities should be achievable using affinity maturation techniques. However, as we discuss below, it may not be desirable to achieve affinities this high since impaired tumor penetration may result.

Other methods for the randomization of protein sequences, such as propagation of the scFv genes in *E. coli* mutator strains (Irving et al., 1996; Low et al., 1996) or DNA shuffling (Stemmer, 1994; Crameri et al., 1996) can also be effective techniques for the affinity maturation of scFv molecules. Finally, novel screening tools other than phage display have been described over the past few years. In particular, ribosomal display (Hanes and Plückthun, 1997; Hanes et al., 1998) has emerged as a technique with significant promise for future development of high affinity reagents.

7. Effective in vivo tumor targeting

A number of factors determines the ability of an antibody-based molecule to target, penetrate and be retained in a tumor expressing its relevant antigen. Some are independent of the antibody, for example, the tumor type (e.g., solid vs. diffuse), the relative abundance of the target antigen in both the tumor and in normal tissues and degree of necrosis of the tumor. However, many properties of the antibody, such as its affinity, size, valence and pI, have a direct effect on the success of its tumor targeting.

7.1. Is more affinity necessarily a good thing?

Significant efforts have recently been expended to select higher affinity scFv through the creation of larger libraries and to enhance scFv affinity using approaches similar to those outlined above. However, there is a growing body of evidence that for some applications (e.g., targeting solid tumors) extremely high affinities actually hinder the ability of antibody-based proteins to penetrate into the tumor from the vasculature. Weinstein first postulated the existence of this phenomenon, which he termed the "binding site barrier" effect (Weinstein et al., 1987; Fujimori et al., 1989). To paraphrase Weinstein, antibodies with an extremely high affinity will bind in an essentially irreversible manner to the first tumor antigen encountered, resulting in antibody localization only in the highly vascularized regions of the tumor. Initial attempts to test this hypothesis compared the tumor penetration of a high affinity

(6×10^{-11} M) anti-tumor antibody with an isotype-matched irrelevant control. In this study, Juweid et al. (1992) found that binding of the anti-tumor antibody to its antigen severely limited its penetration into antigen-rich regions of the tumor, while the irrelevant antibody distributed uniformly in these areas. In order to define the role of affinity on tumor retention and penetration, we have recently performed biodistribution studies in tumor bearing *scid* mice using the series of anti-HER2/*neu* C6.5-based scFv molecules described above. As these scFv molecules span a range of affinity (from $\sim 10^{-7}$ to $\sim 10^{-11}$ M) for the same epitope of HER2/*neu*, a precise evaluation was possible. We found that below a threshold affinity of about 10^{-8} M specific tumor localization was not detectable at 24 h after injection. Increasing the affinity to $\sim 10^{-8}$ and 10^{-9} M resulted in 4-fold and 7.5-fold respective increases in tumor retention (Adams et al., 1998a). Above 10^{-9} M, further enhancements in binding affinity did not translate to increased tumor retention (Adams et al., 1998b). These results are in agreement with the observation by Jackson et al. (1998) that a series of high affinity anti-CEA affinity mutants (8×10^{-9}–6×10^{-10} M) displayed similar tumor retentions at 24 h post injection. Furthermore, in preliminary immunohistochemical studies performed with our affinity mutant scFv molecules we observed that their ability to penetrate solid ovarian carcinoma xenografts overexpressing the target antigen decreased with increasing affinity (unpublished results). Consequently, it appears that expending major efforts to create ultra-high affinity antibody-based molecules for the treatment of solid tumors may be counterproductive.

7.2. Size and valence

As noted above, increasing the valence of an antibody-based molecule can enhance its functional affinity. However, the significant increases in size that are usually associated with the creation of multivalent species can greatly alter the pharmacokinetics and potentially hamper the ability of a molecule to diffuse into the tumor. An inverse relationship exists between the impact of size on systemic clearance and tumor penetration on one hand and quantitative tumor retention on the other (Table 1). Small molecules (e.g., 25 kDa scFv) that are capable of relatively rapid diffusion into solid tumors have molecular weights below the renal threshold for first pass elimination ($\sim$ 65 kDa) (Chang et al., 1975; Maack et al., 1979) and hence are eliminated before significant tumor retention can occur. In contrast, large molecules (e.g., 155 kDa IgG) are not eliminated through the kidneys and therefore display a prolonged residence in circulation. A number of factors including heterogeneous tumor blood flow, heterogeneity of tumor vessel permeability and interstitial hypertension combines to form effective barriers to drug delivery (Jain, 1998). In this environment, smaller molecules exhibit a distinct advantage over larger molecules. For example, in an LS174T tumor xenograft, the vascular permeability is 2-fold greater for 25 kDa molecules (e.g., scFv) than for 155 kDa molecules (e.g., IgG) (Yuan et al., 1995). Once the molecule has successfully left the vasculature, interstitial hypertension will also favor the smaller molecule. Mathematical models predict that intact IgG will diffuse 1 mm in 54 h while a smaller Fab fragment ($\sim$ 55 kDa) will diffuse 1 mm in 16 h (Jain and Baxter, 1988). The penetration of radiolabeled scFv, Fab′, $F(ab')_2$ and IgG forms of the CC49 MAb into s.c., antigen-positive, tumor xenografts growing in immunodeficient mice were examined by quantitative autoradiography by Yokota et al. (1992). In these studies, the IgG was concentrated near blood vessels, the scFv distributed more evenly throughout the tumor and an intermediate (size-dependent) pene-

Table 1
Properties of common antibody-based molecules are presented

Structure	Molecular weight (kDa)	Clearance	Tumor penetration	Tumor retention
IgG	155	+	+	+ + + +
$F(ab')_2$	100	+ +	+ +	+ + +
Diabody	50	+ + +	+ + +	+ +
scFv	25	+ + + +	+ + + +	+

tration was exhibited by the Fab′ and $F(ab')_2$ molecules. This demonstrated the impact of size (and possibly valence) on the ability of an antibody-based molecule to evenly distribute throughout a tumor over-expressing relevant antigen.

7.3. Charge (pI)

The charge of an antibody or antibody fragment can impact on pharmacokinetics, biodistribution and tumor-targeting properties. Altering the charge or isoelectric point (pI) can change its interaction with cells, including those of the tumor, endothelium, and kidney. For example, the chemical conversion of surface carboxyl groups to extended primary amino groups results in the cationization of a MAb. This alteration led to an increase the rate of systemic clearance (8-fold faster than native) and the rate of endocytosis into targeted tumor cells (7-fold greater than native), possibly due to increased attractions between the positively charged MAb and the negatively charged tumor and endothelial cells. For proteins that have molecular weights that are near or below the threshold for first pass renal clearance, increasing the pI can accelerate their rate of elimination from circulation via the kidneys. As early as two decades ago, Purtell et al. (1979) observed that modification of the pI of albumin from 4.9 to 7.2–8.2 increased renal excretion by a factor of 300. Recently, Kobayashi et al. (1999) elegantly examined the impact of pI on pharmacokinetics and biodistribution of a humanized anti-TAC Fab fragments in tumor-bearing nude mice. In these studies that employed nonglycolated Fab (pI > 9.3), mildly cationic glyco-Fab (pI = 7–9.3), mildly anionic glyco-Fab (pI = 4.5–7) and strongly anionic glyco-Fab (pI < 4.5), renal accumulation was found to increase significantly as the pI increased. As a result of the lower renal accumulation, the anionic forms of anti-TAC Fab displayed a prolonged residence time in the blood, which in turn led to increased tumor and organ retention. Since the renal accumulation of cationic peptides and small proteins (e.g., Fab fragments) results from retention in the negatively-charged basement membrane of the glomerulum and subsequent tubular reabsorption and lysosomal degradation, strategies have been developed to block this process with an excess of cationic amino acids (e.g., lysine) (reviewed in Behr et al., 1998). Together, these studies indicate that charge modification of antibody-based proteins can be rationally employed to alter pharmacokinetics and targeting.

7.4. Structure and orientation

The structure and orientation of an engineered antibody fragment can have a large impact on its ability to bind to cell surface antigens. For instance, we have found that an anti-HER2/*neu* scFv dimer constructed through the formation of a disulfide bond between very flexible C-terminal-Gly_4Cys tails exhibited a 1.6-fold greater 24 h tumor retention in *scid* mice than a similarly constructed scFv dimer with a shorter, less flexible-SerCys tail (Adams et al., 1993). However, when we employed a second scFv that targeted a different epitope on the HER2/*neu* antigen, the more compact/rigid diabody structure exhibited 3-fold greater 24 h tumor retention than a gene-fused dimer with a flexible $(Gly_4Ser)_3$ spacer (Adams et al., 1998c and unpublished data). Furthermore, raising the affinity of the scFv that comprise the diabody from 10^{-7} to 10^{-9} M did not increase the retention in tumor (unpublished data). Besides having a decreased flexibility, the diabody structure naturally orients the scFv binding sites at opposite ends of the complex, potentially favoring the binding of epitopes that are present on the sides of two proximal cell surface antigens. Clearly, the optimal structure may vary from antibody to antibody.

8. Summary

The recent successes of antibody-based therapeutics for the treatment of cancer have opened the way for a whole generation of similar compounds. As noted above, the optimal characteristics of these molecules has yet to be determined. This is particularly true of MAb-based compounds for the treatment of solid malignancies. At present, the necessity remains for rigorous, head to head preclinical evaluations of panels of reagents with differing characteristics (size, valence, affinity, charge, etc.) but identi-

cal specificity. It is only in this manner that the optimal candidates will be identified for clinical development. The recent advances in the field of antibody engineering should greatly facilitate this process.

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